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The Sendai virus V protein is a nonstructural *trans*-frame protein in which a highly conserved cys-rich Zn^{2+}-binding domain is fused to the N-terminal half of the P protein via mRNA editing. Using a recently developed system in which infectious virus is recovered from cDNA, we have engineered a virus in which a translation stop codon was placed at the beginning of the V ORF. Translation of the V^{stop} mRNA yields a W-like protein, i.e., a protein composed of the N-terminal half of the P protein alone which is naturally expressed at low levels from the P gene. This V-minus but W-augmented virus was found to replicate normally in cell culture and embryonated chicken eggs. The Sendai virus V protein is thus an accessory protein, and the cys-rich Zn^{2+}-binding domain is likely to function in a specialized role during virus propagation.

Sendai virus (SeV) is a prototypic paramyxovirus, containing a nonsegmented negative-strand RNA genome. SeV particles are composed of a helical nucleocapsid core containing the genome RNA of 15,384 nt assembled with 2564 copies of the N (formerly NP) protein, to which ca. 300 copies of the P phosphoprotein and ca. 50 copies of the L (large) protein are bound. This core is surrounded by a lipid envelope containing the F and HN transmembrane glycoproteins and the M (matrix) protein. Paramyxovirus genomes serve as templates for monocistronic mRNAs and for a full-length complementary antigenome strand, the intermediate in genome replication. The synthesis of both genomes and antigenomes is coupled to their assembly, and they are consequently found only as nucleocapsids assembled with N protein (reviewed in Lamb and Kolakofsky, 1996). Six mRNAs (in the order N, P, M, F, HN, and L) are transcribed from the N:RNA genome template by the P-L polymerase. All these viral mRNAs except the P gene mRNA express a single primary translation product from a single ORF. The paramyxovirus P gene mRNAs, in contrast, generally contain alternate ORFs which overlap the N-terminus as well as the middle region of the P protein ORF and express several proteins; e.g., for SeV, the C protein ORF overlaps the N-terminal region of P ORF and is accessed via ribosomal choice during translational initiation (Curran and Kolakofsky, 1990; cf. Fig. 1). The highly conserved, cysteine-rich V ORF which overlaps the middle of the P ORF, on the other hand, is accessed by a mechanism involving transcriptional choice, referred to as cotranscriptional mRNA editing (Vidal and Kolakofsky, 1990).

The *Paramyxovirinae* are currently organized in three genera, the SeV group (including parainfluenza virus type 3 (PIV3)), morbilliviruses (e.g., measles and the distemper viruses), and rubulaviruses (e.g., mumps and SV5). Most of these viral P genes contain an A_nG_n purine run at the start of the internal, overlapping V ORF (Thomas *et al.,* 1988; Cattaneo *et al.,* 1989). The alternate base-pairing possibilities of this "slippery sequence" (including G:U bonds) are used to synthesize *trans*-frame proteins (i.e., proteins whose N- and C-terminal portions are encoded by separate ORFs, like the HIV gag-pol polyprotein (Kollmus *et al.,* 1996). This situation is analogous to ribosomal frameshifting which forms the HIV gag-pol polyprotein, except that for paramyxovirus mRNA editing, the alternate base-pairing occurs during mRNA synthesis, via pseudo-templated transcription (Jacques and Kolakofsky, 1991). As a result, mRNAs with expanded G runs are transcribed from these genes in addition to those which are faithful copies of their templates, and the number of G insertions which occur for each virus group mirrors their requirements to switch between the in-frame and the out-of-frame ORFs (reviewed in Kolakofsky *et al.,* 1993). For the morbilliviruses and some of the SeV group, which require a +1G insertion to access the V ORF from the genome-encoded P ORF (Fig. 1), a single G is added as the predominant insertional event, generating a V mRNA coding for the *trans*-frame V protein. Two Gs, however, are also inserted at a much reduced frequency, and this leads to a protein called W, which is essentially the N-terminal part of the P protein up to the editing site (this downstream ORF is quickly closed by a stop codon; Fig. 1). For the rubulaviruses, in contrast, P is the *trans*-frame protein and these P genes require a 2G insertion at the editing site to access the remainder of the P ORF from the genome-encoded V ORF, and 2Gs are added here at high frequency when insertions occur. The remaining P gene protein of rubulaviruses, representing the N-terminal part of the V or P protein up to the editing site (and referred to [with tongue in cheek] as the I protein), is

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accessed by the insertion of 4 Gs rather than 1 G residue (Paterson and Lamb, 1990).

The paramyxovirus P proteins are modular proteins whose domains are shuffled via mRNA editing (Curran *et al.,* 1991). The best studied of these domains lie in the C-terminal 40% of the SeV protein. When SeV mRNA synthesis is studied independent of genome replication, only those regions which lie downstream of the G insertion site (codon 317) are required, namely, the predicted coiled-coil responsible for the protein's trimerization, the stable L protein binding site, and the N:RNA binding site (Curran, 1996) (Fig. 1). This C-terminal 40% of the SeV P protein thus represents the minimal polymerase module. The predicted coiled-coil and the putative N:RNA binding site (a predicted triple α-helical bundle), moreover, appear to be conserved at the same relative positions for all the *Paramyxovirinae* (Curran *et al.,* 1995); i.e., the RNA synthesis (or minimal polymerase) region is always located in the C-terminal half of P. In SeV and the morbilliviruses, mRNA editing therefore serves to detach this minimal polymerase domain from the N-terminal half of the P protein and either to replace it with a cysteine-rich Zn^{2+}-binding domain of yet unknown function or to simply truncate the protein at this point. For rubulaviruses, on the other hand, mRNA editing carries out the converse function, to replace the cysteine-rich domain with the minimal polymerase region, or to truncate the protein at this point. To date, only a single functional domain, for chaperoning unassembled N protein during the nascent chain assembly step of genome replication (aa 33–41, Fig. 1), has been mapped to the N-terminal 60% of the SeV P protein (Curran *et al.,* 1995). Its location is consistent with the finding that overexpression of V or W can inhibit genome replication, but not mRNA synthesis (Curran *et al.,* 1991). The only other known feature of the N-terminal part of SeV P is that it contains the site(s) at which phosphates are added in a stable fashion (Byrappa *et al.,* 1995, 1996).

The paramyxovirus V proteins are remarkable not only because they (or their P proteins partners) are *trans*-frame proteins, but also because the V ORF is so well conserved in a gene whose other ORFs are so poorly conserved. Each V ORF contains 7 invariant cysteines which coordinate two atoms of Zn^{2+} (Liston and Breidis, 1994; Paterson *et al.,* 1995), and there are a further 7 invariant residues, all within a span of some 60 residues. The function of this structural motif, however, has remained an enigma. For the SeV group and the morbilliviruses, the Zn^{2+}-binding domain is fused to the acidic N-terminal half of P (250–320 residues), and these proteins are not stably associated with nucleocapsids, nor are they found in virions (Curran *et al.,* 1991; Wardrop and Breidis, 1991; Gombart *et al.,* 1992; Liston *et al.,* 1995). For the rubulaviruses, on the other hand, the Zn^{2+}-binding domain is fused to the shorter N-terminal half of P (ca. 160 aa), which is basic in nature and which is stably associated with nucleocapsids and well represented in virions (Thomas *et al.,* 1988; Takeuchi *et al.,* 1990; Precious *et al.,* 1995). It is therefore possible that these different V proteins carry out different functions in the various paramyxoviruses, even though they carry similar Zn^{2+}-binding domains.

This paper reports that a SeV genetically engineered to express a W-like protein in place of the V protein is viable. Moreover, this ablation of V ORF expression confers neither a selective advantage nor a disadvantage in infections of cell lines in cultures or in embryonated chicken eggs. This V protein therefore appears to serve a specialized or accessory function during virus replication.

METHODS AND MATERIALS

Cells and viruses

HeLa, LLC-MK2, BHK, and MDCK cells were grown in MEM medium supplemented with 5% fetal calf serum. Wild-type and recombinant Sendai virus stocks were prepared in the allantoic cavity of 9-day-old embryonated chicken eggs. Stocks were harvested after 3 days at 33°, clarified, and stored at −70°. Titration and plaque isolation of transfectant viruses were carried out in LLC-MK2 cells covered with a 0.3% agarose overlay containing 1.2 μg/ml acetyl-trypsin, for 3–4 days at 33°.

Construction of the V-SeV cDNA

The *Sma*I fragment from the SeV P gene (nt 2760–3552) was first subcloned into pGEM-4. Two sets of PCR were realized, SP6 with 5′ CATAGGAGATAACACATC-ATC (nt 2793–2814) and T7 with 5′ GATGATGTGTTA-TCTCCTATG (complementary strand), followed by a fusion PCR (SP6 with T7) such that a stop codon (TAA) was introduced in the V ORF (Fig. 1). The *Sma*I fragment was then introduced into pGEM-P/C^{stop} to generate pGEM-P/C^{stop}/V^{stop} and into pFL3 to generate pFL3-V^{stop}.

Recovery of recombinant Sendai viruses

Transfection experiments were carried out as described previously (Curran *et al.,* 1991). HeLa cells in 9-cm-diameter dishes were infected with the recombinant vaccinia virus vTF7-3 (Fuerst *et al.,* 1986) at a m.o.i. of 3. At 1 hr postinfection, cells were washed twice with MEM lacking serum and transfected with a plasmid mixture containing 1.5 μg pGEM-L, 4.5 μg pGEM-N, 4.5 μg pGEM-P, 15 μg pFL3 or pFL3-V^{stop}, and 20 μl homemade TransfectAce (Rose *et al.,* 1991) in 2.5 ml MEM. At 16 hr posttransfection, the cells were washed and the medium was replaced with MEM containing 100 μg/ml AraC. At 48 hr posttransfection, the cells were removed from the dish and injected into the allantoic cavity of 9-day embryonated chicken eggs (Garcin *et al.,* 1995).

The presence of virus in the allantoic fluids was determined by infecting BHK or MDCK cells with a 1/10 dilution of each stock. At different times postinfection, cells

were harvested and lysed with 20 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, and 0.5% NP-40; and the cytoplasmic extracts were centrifuged on 20–40% (w/w) CsCl density gradients to purify the viral nucleocapsids as well as pellet the mRNAs.

Radiolabeling and immunoprecipitation

BHK cell monolayers (5 cm) were infected at a m.o.i. of about 10 with wild-type or recombinant Sendai viruses. At different times postinfection, cells were pulse-labeled for 30 min with 100 μCi/ml of ^{35}S-Translabel (NEN) in methionine-free medium. Cells were then solubilized in 500 μl of lysis buffer (50 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 10 m*M* EDTA, 0.6% NP-40, 1 m*M* DTT), after which nuclei were removed by pelleting at 12,000*g* for 10 min. One hundred microliters of the cytoplasmic extracts were used for immunoprecipitation. Immune complexes were recovered with protein-A–Sepharose. Beads were washed three times with lysis buffer and selected proteins were analyzed by SDS–PAGE.

Primer extension methods

The 5′ end-labeled primers (1 pmol) were coprecipitated with either RNAs from purified nucleocapsids [primer extension with 5′ CGGCCATCGTGAACTTTGGC (nt 106–126) and 5′ GAAGCTCCGCGGTACC (nt 15270–15286)] or pellet RNAs (limited primer extension) with 5′ GATGTGTTCTCTCCTATG (nt 2793–2811). Primer extension protocols were carried out as described previously (Garcin and Kolakofsky, 1990; Pelet *et al.,* 1991). The SeV genome is 15,384 nt long.

RESULTS

Isolation of a V-minus but W-augmented rSeV

To generate a virus without the ability to express a cysteine-rich Zn^{2+}-binding domain, we introduced a TAA stop codon in the V ORF just downstream of the mRNA editing site of FL3, by mutating G^{1063} to T (Fig. 1), generating FL3-V^{stop}. In a hypothetical rSeV-V^{stop} infection, ribosomes translating P gene mRNAs with a single G insertion would thus terminate on the fourth codon downstream, after shifting from the P into the V ORF. This generates a protein referred to as W* [it is identical to W except that the C-terminal ala has been replaced by the tripeptide his-arg-arg (Fig. 1)]. Ribosomes translating mRNAs without G insertions would generate a P protein (P^{D320}) with a single aa substitution: glu^{320} (GAG) replaced by asp (GAT) (underlined in Fig. 1). The G^{1063}/T mutation was also introduced into pGEM-P/C^{stop}, the support plasmid generating the P protein that initiates virus recovery and whose C protein ORF is closed by a stop codon. C protein expression is highly detrimental to virus recovery and must be suppressed for this process (Cadd *et al.,* 1996). rSeV recovery from FL3 was found to be as efficient using pGEM-P^{D320}/C^{stop} as pGEM-P/C^{stop}, indicating that the glu^{320}/asp substitution had little or no effect on P protein function (data not shown).

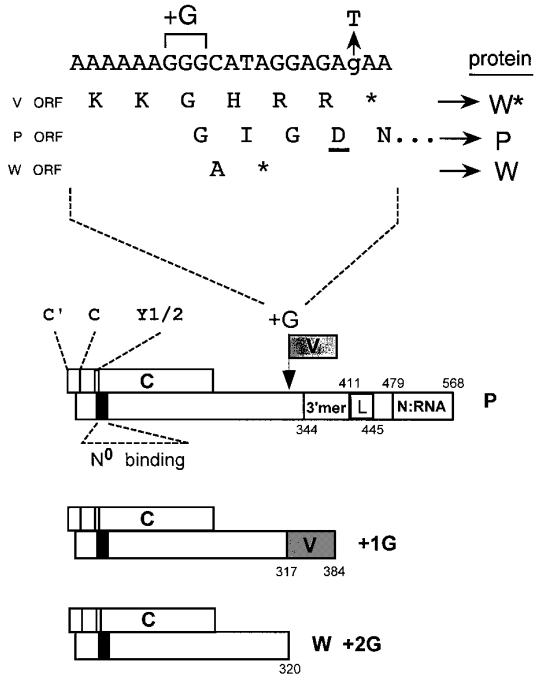

FIG. 1. The ORFs of the SeV P gene. The C, P, and V ORFs expressed by the SeV P gene are shown as boxes drawn roughly to scale. C′, C, Y1/2 refer to the four ribosomal initiation sites of the C proteins. The boxes within the P ORF indicate identified domains: the domain involved in chaperoning unassembled N protein (N^0) for the nascent chain assembly step of genome replication (aa 33–41), the predicted coiled-coil trimerization domain (3′mer; aa 344–411), the L protein binding site (aa 411–445), and the domain for binding to the template (N:RNA; aa 479–568). The mRNA editing, or G insertion, site is indicated with an arrow, and the nucleotide sequence of this region and the three ORFs opened downstream of this site are also shown (in single-letter code) at the top. The single point mutation used to close the V ORF (G^{1063}/T) is indicated, as is the resulting E-to-D substitution (underlined) in the P ORF. The organization of the expressed ORFs of the 1G (V/W*) and 2G (W) inserted mRNAs is shown at the bottom.

FL3-V^{stop} DNA was then transfected into cells along with pGEM-P/C^{stop}/V^{stop}, and as a positive control, FL3 DNA was transfected into cells along with pGEM-P/C^{stop}. This recovery system is highly recombinogenic, and mutations in the FL3 P gene should be matched with those in pGEM-P (especially if the mutation confers a selective disadvantage), to avoid the loss of the mutation by recombination. The transfected cells were then injected into embryonated chicken eggs, and the second-passage allantoic fluids were used to infect BHK cells. The infected cultures were labeled with ^{35}S-Translabel, and cell extracts were precipitated with specific antibodies. Virus was apparently recovered from both FL3-V^{stop} and FL3 DNA, as normal amounts of N and P proteins (relative to infection with natural SeV^H) were found in both infected cultures (Fig. 2a). Virus recovered from FL-3 DNA also expressed a V protein similar to that produced by SeV^H-infected cells, whereas V protein could not be detected in the infection with virus recovered from FL-$3^{V\text{-}stop}$ DNA

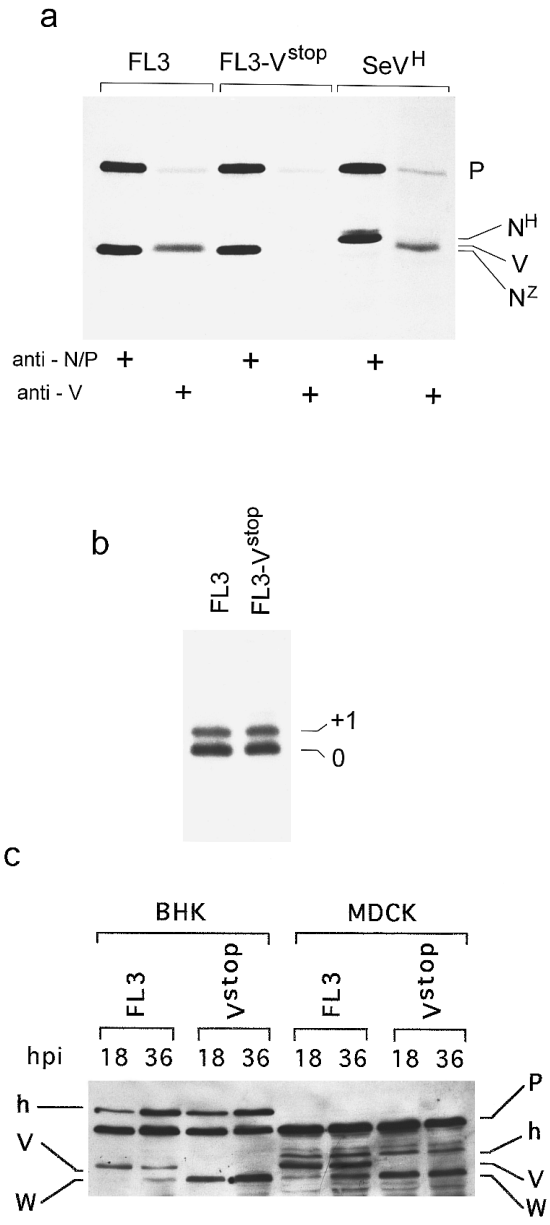

FIG. 2. P gene expression of rSeV-V^{stop}. (A) BHK monolayers were infected with a 1:10 dilution of the second-passage egg allantoic fluid of virus recovered from FL3 and FL3-V^{stop} DNA, as well as natural SeV^{H}. The cells were labeled with 50 μCi ^{35}S-Translabel from 12 to 24 hr postinfection. Cytoplasmic extracts were prepared and reacted either with a mixture of monoclonal antibodies to N and P or with a rabbit polyclonal antiserum to a V peptide. The immunoselected proteins were separated by SDS–PAGE (10%) and detected by autoradiography. (B) CsCl pellet RNA was prepared from BHK cells infected with virus recovered from FL3 and FL3-V^{stop} DNA. The distribution of mRNAs with insertions at the editing site was determined by limited primer extension with a primer whose 3′ end abuts the A_6G_3 purine run, and ddATP was used to terminate cDNA synthesis beyond the purine run. The products were separated on a 10% sequencing gel. Zero and +1 mark the positions of the uninserted and +1G inserted mRNAs. The +2 band is not visible at this exposure. (C) BHK and MDCK cells were infected with 10 PFU/cell of virus recovered from FL3 DNA or that from FL3-V^{stop} DNA which was *Nsi*-I^{R} (listed as V^{stop} above). The cells were harvested at 18 and 36 hpi, as indicated. Cytoplasmic extracts were prepared, separated by 10% SDS–PAGE, and examined by immunoblotting with a rabbit polyclonal antiserum to the entire P protein (anti-P^{SDS}). "h" refers to host protein bands.

(Fig. 2a). On the other hand, when infected cell extracts were immunoprecipitated with polyclonal rabbit anti-P antiserum (which detects the P, V, and W proteins), the absence of V in the V^{stop} extracts is now found to be compensated for by the increase in the level of W protein (Fig. 2c), as expected. The virus stock recovered from FL3-V^{stop} DNA (rSeV$^{V-/W++}$) was further characterized by RT/PCR amplification of nt 21–126 of the leader/N gene junction [where the *Bgl*II and *Nsi*I markers are located (Garcin *et al.,* 1995)] and nt 2760–3552, which carries the point mutation of the P gene. All of the PCR nt 21–126 fragment could be digested with *Bgl*II, and part could be digested with *Nsi*I [as found previously (Garcin *et al.,* 1995)], indicating that the virus stock was derived from FL3 DNA. The amplified P gene fragment was also found to have retained the G^{1063}/T mutation, consistent with the undectable amounts of V protein and increased levels of W, in rSeV-V^{stop} (rSeV$^{V-/W++}$) infected cells.

We also examined whether the inability of rSeV-V^{stop} to express the V ORF affected the cotranscriptional editing of the P gene mRNAs, by using by limited primer extension (Methods and Materials) to determine the fraction of these mRNAs which contained insertions at the editing site. As shown in Fig. 2b, virus recovered from FL3-V^{stop} behaved identically to that recovered from FL3 in this respect, in that ca. 25% of these mRNAs contained a single G insertion and $<$5% contained a 2G insertion. The cotranscriptional editing of the P gene mRNA therefore does not appear to be regulated by the product of the V ORF.

Heterogeneity in the rSeV-V^{stop} stock

The marker *Nsi*I site in FL3 introduces an upstream, out-of-frame ribosomal initiation site in the N gene mRNA (AGTATGCAT), and several-fold less N protein is expressed from this N mRNA (Garcin *et al.,* 1995). DNA amplified by RT-PCR from the early passage FL3-derived rSeV stocks is often partially digested with *Nsi*I, indicative of heterogeneity in the early passage virus stocks. Until these experiments, cleavability by *Nsi*I was routinely lost on further passage of the stocks in eggs (consistent with the selective disadvantage of virus with lowered N protein expression), and *Nsi*I-sensitive (*Nsi*-I^{S}) DNA was generally not detectable by passage 4. The fraction of *Nsi*-I^{S} DNA amplified from virus derived from FL3-V^{stop} DNA, however, did not diminish on subsequent passage and was stable to at least passage 7. Virus clones were therefore isolated from this stock by plaquing, and nt 21–126 of their genomes were amplified by RT-PCR. Digestion of these DNAs with *Nsi*I showed that both *Nsi*-I^{S} virus and *Nsi*-I^{R} virus were present in the original virus stock, and representatives of the *Nsi*-I^{S} and *Nsi*-I^{R} clones were then sequenced. The *Nsi*-I^{R} clones were found to contain the sequence AGTACGCAT, whereas those that had remained *Nsi*-I^{S} contained TGTATGCAT at this site.

We previously found that rSeV which had mutated its

*Nsi*I site to AGTGTGCAT had apparently reestablished normal N protein expression levels, as this virus could compete equally with rSeV containing the wild-type sequence at this site (AGTATCCAC) in mixed infections of eggs (Garcin *et al.,* 1995). Non-ATG ribosomal start codons might be used very sparingly at this site due to the presence of cytosine at position +4 relative to the start codon (e.g., AGTACGCAT) (Kozak, 1989). It is possible that the rSeV-V^{stop}/*Nsi*-I^{S} has also reestablished normal N protein expression levels even though the ATG start codon remains in place, because in this context (TGTATGCAT) there is neither a G at position +4 nor a purine at position −3. To examine the relative efficiencies with which the two rSeV-V^{stop} viruses expressed N protein, equal amounts (in PFU and total virus protein, cf. Fig. 4) of rSeV-V^{stop}/*Nsi*-I^{S}, rSeV-V^{stop}/*Nsi*-I^{R}, and as a control, rSeV^{H} (with wt sequence at this site) were used to infect BHK cells. These cells were then pulse-labeled with ^{35}S-Translabel for 45 min at different times postinfection, and the amounts of N proteins synthesized during these times (relative to those of P and L) were determined (by precipitation with specific antibodies, followed by SDS–PAGE). We found that at 20 and 48 hr postinfection (when mRNAs levels are at equilibrium), equal amounts of the N, P, and L proteins were made by all three viruses (not shown). The AGTACGCAT and TGTATGCAT mutations of the rSeV-V^{stop} viruses thus both appear to have effectively eliminated the out-of-frame ribosomal initiation at this site, thus relieving the inhibition of translational initiation at the *bona fide* downstream N protein start site.

Absence of V ORF expression has little effect on virus multiplication in cultured cells

We compared the infection of BHK, HeLa, LLC-MK2, and MDCK cells in culture with equivalent amounts of rSeV-V^{stop} and rSeV^{H}, by examining (i) cytopathogenicity by light microscopy, (ii) the accumulation of viral proteins by immunoblotting or their rates of synthesis by pulse-labeling, (iii) the accumulation of genomes and antigenomes by primer extension analysis, and (iv) the liberation of virus particles by SDS–PAGE (cf. Fig. 4). In all cases, little or no difference in the infection of cells by rSeV-V^{stop} or rSeV^{H} could be found; the accumulation of genomes and antigenomes is shown in Fig. 3, and the accumulation of the P, V, and W proteins in Fig. 2c. Note that the substitution of V expression with that of W did not affect the kinetics of genome amplification nor the ratios of genomes to antigenomes (Fig. 3). The absence of V protein expression also did not affect the cytopathic effects of the infection, the titers of virus liberated, or the final accumulation of viral macromolecules intracellularly.

Absence of V ORF expression has little effect on virus multiplication in embryonated chicken eggs

As mentioned above, when passage 2 (P_2) rSeV stocks composed of an equal mixture of *Nsi*-I^{S}/AUG^{+} (low N protein synthesis) and *Nsi*-I^{R}/AUG^{-} viruses (normal N protein synthesis) are further passaged twice in eggs (P_4), *Nsi*-I^{S}/AUG^{+} genomes become undetectable, presumably because *Nsi*-I^{S}/AUG^{+} viruses are selected against. Moreover, when natural SeV and rSeV, or rSeV-GP42 and SeV, are passaged together in eggs, similar selectivity occurs, as the former virus in each case eliminates the latter in one or two cycles (Garcin *et al.,* 1995). The selectivity found in the mixed-virus infections of eggs

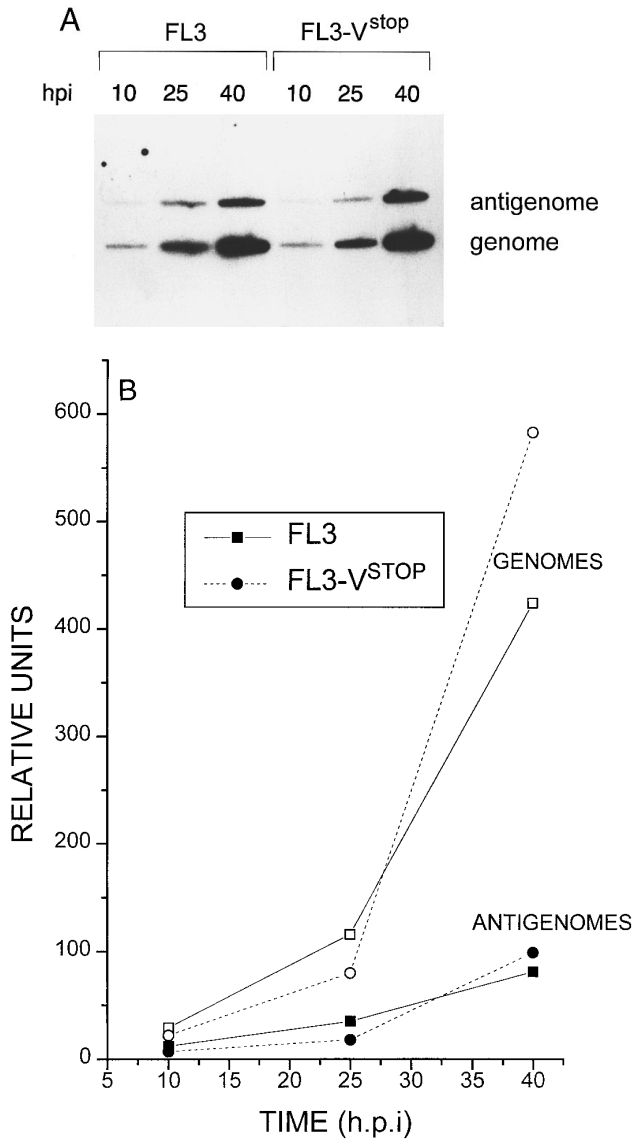

FIG. 3. The accumulation of genomes in rSeV-V^{stop}-infected cells. BHK monolayers were infected with 10 PFU/cell of virus recovered from FL3 or FL3-V^{stop} DNA. Cells were harvested at the various times (hours postinfection) indicated, and their viral nucleocapsids were isolated by banding on CsCl density gradients. The relative amounts of genomes and antigenomes present in these fractions was determined by extending a mixture of genome- and antigenome-specific primers (which are 114 and 126 nt, respectively, away from the 5′ ends of these chains) on equal fractions of the RNAs recovered from the banded nucleocapsids. The extended products were separated on a 10% sequencing gel (A). Their relative amounts were determined in a PhosphorImager and are plotted in B.

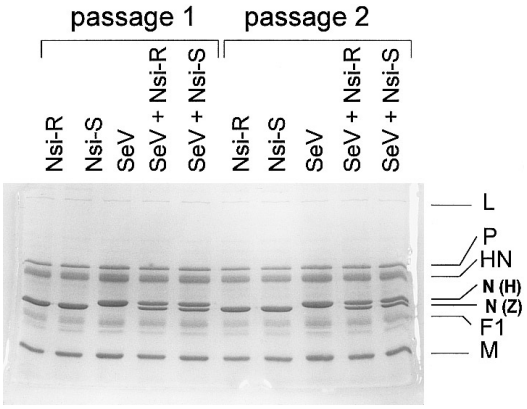

FIG. 4. Competition of rSeV-V^{stop} and rSeV in embryonated chicken eggs. Virus clones having lost (*Nsi*-R) or retained (*Nsi*-S) the marker *Nsi*I site of FL3 were isolated by plaquing from the fourth-passage allantoic fluid of rSeV-V^{stop} (see text). One clone from each of the rSeV-V^{stop} viruses, along with rSeV^{H} (SeV), was amplified by a further passage in eggs. All three viruses produced allantoic fluids of similar titers (2×10^8 PFU/ml), with equivalent amounts of viral proteins as determined by SDS–PAGE and Coomassie brillant blue staining of viral proteins pelleted from 50 μl of allantoic fluid. A 1:1000 dilution of the *Nsi*-R, *Nsi*-S, and rSeV^{H} stocks, as well as 1:2000 dilutions of *Nsi*-R plus rSeV^{H}, and *Nsi*-S plus rSeV^{H}, was amplified in eggs (passage 1). Virus was pelleted and equivalent amounts (50 μl of allantoic fluid) were used to estimate the relative yield of each virus. Each allantoic fluid was then passaged in eggs after a 1:1000 dilution (passage 2) and virus yield was again estimated, as above. The viral proteins, including the H and Z electrophoretic forms of the N protein, are indicated on the right; the unmarked band just below F1 is presumably cellular actin.

is presumably due to the multiple rounds of virus growth which take place here and the relative advantage in growth of one virus over another. We therefore similarly examined whether co-infecting eggs with equivalent amounts of rSeV-V^{stop} and rSeV^{H} led to the preferential amplification of one of the two viruses. rSeV-V^{stop} expresses the Z strain N protein (N^{Z}) and rSeV^{H} expresses that of strain H (N^{H}), and the electrophoretic mobilities of these two N proteins are distinguishable (Fig. 4). The relative presence of each viral genome intracellularly (or liberated as virus) in co-infections can then be determined by simply examining the viral proteins by SDS–PAGE. We found that eggs co-infected with equivalent amounts of rSeV$^{V\text{-}stop}$ and rSeV^{H} gave rise to equivalent amounts of each virus progeny and that further passage of this stock in eggs did not alter the result (Fig. 4). Infection of embryonated chicken eggs individually with each rSeV also gave rise to stocks with equivalent titers. Similarly, co-infection of cells in culture did not favor rSeV$^{V\text{-}stop}$ or rSeV^{H}. We were therefore unable to detect a selective advantage or disadvantage of virus which does not express a P gene protein containing a cys-rich Zn^{2+}-binding domain, either in cells in culture (single cycle of infection) or in embryonated chicken eggs (multiple cycles of infection).

DISCUSSION

P gene mRNA editing leads to the synthesis of two truncated proteins. Each contains the N-terminal 317 aa of the P protein and then either a highly conserved cys-rich Zn^{2+}-binding domain (68 aa V ORF) or 2 aa from the remaining ORF (W protein). Both the V and the W proteins inhibit genome replication (but not transcription) in a dose-dependent manner (Curran *et al.,* 1991, 1994). This inhibitory effect is then presumably due to the common N-terminal 317 aa of the P protein, rather than the cys-rich domain of V. The N-terminal half of P is not required for mRNA synthesis, but it is required for genome replication, where it is thought to function in the concurrent assembly of the nascent genome chain into nucleocapsids. Both V and W interact with unassembled N protein (Curran, unpublished results), and this could explain the specific inhibition of replication observed in their presence. Moreover, Kato *et al.* (in press) have recently engineered a SeV which cannot edit its P gene mRNA and therefore expresses neither the V nor the W proteins (rSeV$^{V-/W-}$). While infection of mice with rSeV$^{V-/W-}$ is highly attenuated, its infection of some cultured cells is potentiated; i.e., rSeV$^{V-/W-}$ replicates faster and is more cytopathic. The faster replication of rSeV$^{V-/W-}$ in some cell lines is consistent with the notion that V and W act to slow genome replication during infection of some cells. Interaction between the V and the N proteins has also been reported for SV5 (Precious *et al.,* 1995) and measles virus (Liston *et al.,* 1995), although in these cases it is unclear whether these interactions map to the cys-rich region of V.

All *Paramyxovirinae* contain a minimum of six genes (N, P, M, F, HN, and L, and invariably in this order) which represent the minimal essential genes of this virus subfamily. Each of the *Paramyxovirinae* also expresses at least one other ORF from their P genes. The C ORF which overlaps the N-terminus of the P ORF is expressed by all the SeV group viruses and the morbilliviruses, but the rubulaviruses do not contain an overlapping ORF here. The relatively short V ORF which overlaps the middle of the P ORF is expressed by all the *Paramyxovirinae* except for hPIV1 and hPIV3 (both members of the SeV group) (Matsuoka *et al.,* 1991; Galinski *et al.,* 1992; Thierry Pelet, unpublished results). The SeV group (paramyxovirus genus) is made up of two pairs of highly related viruses: human and murine PIV1 (SeV), and human and bovine PIV3. Neither of these human parainfluenza viruses expresses a V protein, in contrast to their nonhuman pairs. For hPIV1, in particular, there is no discernible mRNA editing site, and the remnants of the V ORF (68 residues long) have been closed by 9–11 stop codons, hence this virus cannot express a V (or a W) protein (Rochat *et al.,* 1992). The virus most closely related to SeV in evolution can clearly do without a V protein. hPIV1, however, cannot grow in certain cell lines in which SeV can replicate well, and this restriction appears to lie at the level of genome amplification (Tao and Ryan, 1996). It will be of interest to examine whether the growth of rSeV-V^{stop} (rSeV$^{V-/W++}$) is attenuated in these same cell lines. There are two possible explanations why the V ORF is ex-

pressed by almost all, but not all, of the *Paramyxovirinae.* These P gene proteins may be accessory proteins, in that they are not absolutely required by all the viruses (in contrast to the minimal essential proteins), and some viruses have adapted to do without them, e.g., hPIV1. The other possibility is that the function of V is always essential, but in some cases it has been transferred elsewhere, e.g., to one of the minimal essential proteins. Our finding that the ablation of the zinc-binding domain of the SeV V protein neither helps nor hinders the infection of cultured cells suggests that this domain is only conditionally required, i.e., that V is an accessory protein (Trono, 1995). Similar conclusions have been drawn from the finding that ablation of the measles virus and vesicular stomatitis virus C proteins similarly has little or no adverse effect on virus multiplication in cell culture (Radecke and Billeter, 1996; Kretzschmar *et al.,* 1996).

We were unable to show an effect of the absence of V expression in the rSeV-V^{stop} infection, even in chicken eggs in which multiple rounds of replication occur and in which SeV grows to such high titers (10^9 PFU/ml). Although this gives us little information about the function of V, these same experiments provide the evidence for just how slight the effect of substituting V expression by that of W has in the culture systems we have investigated. These results point to a specialized role for the cys-rich domain of V during infection, and one that is not required for (nor harmful to) cellular infections. Laboratory strains like SeV^H and SeV^Z have been maintained in eggs since the early 1950s and thus have been passaged many times in this culture system. Mutations which close the V ORF may very well have arisen during this time, but as they would have no selective advantage or disadvantage, they would presumably be found as just another quasi-species and would not have been noticed (Holland *et al.,* 1992). This situation is in marked contrast with that reported for dolphin morbillivirus (DMV) (Bolt *et al.,* 1995). When virus from infected dolphins is grown in cell culture, variant genomes with P gene editing sites changed from GGG to GAG [such that they presumably cannot edit their P mRNA (Vidal *et al.,* 1990) and therefore cannot produce either V or W proteins] were readily detected. By the above criterion, this $DMV^{V-/W-}$ genome may be selected for in cell culture, but selected against in its animal host.

The strong conservation of the V ORF throughout almost all of the *Paramyxovirinae* suggests that it may interact with a structurally invariant surface, e.g., a cellular protein which is highly conserved from chickens (Newcastle disease virus) to man. Interactions between V and cellular proteins have been reported for measles virus (Liston *et al.,* 1995) and SV5 (Precious *et al.,* 1995), although cellular proteins of very different sizes (150 kDa for SV5, and 17, 38, and 60 kDa for measles) were noted. The absence of a phenotype for rSeV-V^{stop} ($rSeV^{V-/W++}$) in cell culture is consistent with the notion that this interaction with cellular proteins may only manifest itself in animal infections, and the attenuated phenotype of $rSeV^{V-/W-}$ in mice is particularly noteworthy in this respect (Kato *et al.,* in press). Following normal virus titers in the lungs for the first 2 days, titers of $rSeV^{V-/W-}$ progressively decrease (in contrast to normal $rSeV^{V+/W+}$), the infection is cleared, and all the mice survive. V expression therefore appears to act in animal infections to avoid the antiviral response of the host (Kato *et al.,* in press). It will be of interest to determine whether the infection of mice with rSeV-V^{stop} ($rSeV^{V-/W++}$) is similarly attenuated. It remains possible that the attenuated phenotype of $rSeV^{V-/W-}$ in mice is due (at least in part) to abnormally rapid viral gene expression intracellularly which alerts the host viral defense mechanisms too quickly, helping the animal to overcome the infection.

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